A3 TRANSLATIONAL PHARMACOLOGY STRATEGY TO EVALUATE MMP-13 INHIBITORS FOR THE TREATMENT OF OA; USE OF BIOMARKERS

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Purpose: Demonstrating efficacy of potential disease-modifying OA drugs (DMOAD) requires long treatment times and a large number of subjects, resulting in expensive clinical trials. An important risk of such studies is the absence of beneficial effect of the drug. Accurate prediction of the doses that would result in cartilage protection and joint preservation would help minimize this risk. Although dose may be extrapolated from pre-clinical studies, the lack of approved DMOADs obscures the clinical predictive capability of the OA animal models. Here we describe a translational pharmacology strategy using TIINE (a type II collagen neoepitope assay) to evaluate MMP-13 inhibitors for the treatment of OA
Methods: The MMP-13 inhibitors belong to the class of compounds that bind the S_1' pocket of the enzyme providing good potency (Ki 2−6 nM) and selectivity (>4000 fold over 15 other MMPs-, ADAMTS-4 and -5, and TACE). TIINE was measured using a sandwich immunoassay and an LC-MS/MS assay specific for the 45-mer peptide. Rat medial meniscal tear (MMT) and dog partial medial meniscectomy (pMx) were performed at Bolder BioPath as previously described. All studies were approved by the Institutional Animal Care and Use Committees.
Results: Studies in vitro showed that TIINE was generated by addition of active MMP-13 to de-vitalized cartilage in a time- and concentration-dependent manner, which was inhibited by MMP-13 inhibitors. Because TIINE was also generated by addition of other MMPs, this biomarker is not specific for MMP-13 activity. In vivo, TIINE was upregulated about two fold in the urine from OA patients in contrast to age-matched controls. To understand the role of MMP-13 in the generation of such OA-associated TIINE elevation, TIINE modulation was evaluated in animal models of OA, and in response to MMP-13 selective inhibitors. In the rat MMT, TIINE was elevated in the synovial fluid (SF) from the operated knee, but not the contralateral knee, and it was inhibited in a time- and dose-dependent manner following oral administration of an MMP-13 inhibitor to a maximum of ~50%. Similar TIINE inhibition in the SF and urine was observed at the end of a 4-wk study (BID, prophylactic), and maximal inhibition correlated with cartilage protection as evaluated by histology. All animals and human subjects evaluated to date excrete some amount of TIINE in their urine (which is age-dependent). Single dose oral administration of MMP-13 inhibitor to skeletally-mature beagle dogs (naive) inhibited baseline urinary TIINE in a time- and dose-dependent manner up to ~50%. In the 4-wk dog pMx model, MMP-13 inhibitors (BID, prophylactic) protected cartilage degeneration at exposures that inhibited TIINE ~45% at 2, 3 and 4 wks. These results suggest that doses that result in ~45% inhibition of TIINE in early clinical trials may protect cartilage degradation and joint structure in Phase 2−3 studies. In previous clinical studies with broader spectrum MMP inhibitors, urinary TIINE was found to be inhibited in both human healthy volunteers and OA patients.
Conclusions: The proposed translational pharmacology strategy includes: (1) determine drug plasma and/or SF concentration required for maximum TIINE inhibition in urine of naive and/or OA animals, (2) establish correlation between TIINE inhibition and cartilage protection in animal models of OA, (3) evaluate TIINE inhibition in healthy volunteers and/or OA patients in early clinical trials, and (4) in POC studies, use the doses that resulted in sufficient inhibition of TIINE from the FIH studies and that was associated with cartilage protection in pre-clinical studies.

A4 HYPOXIA-INDUCIBLE FACTOR 2A (HIF2A) CONTROLS SEQUENTIAL STEPS IN THE LATE STAGE OF ENDOCHONDRAL OSSIFICATION

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Purpose: The late stage of endochondral ossification including chondrocyte hypertrophy, cartilage matrix degradation, and vascular invasion are known to be crucial not only in physiological skeletal growth, but also in cartilage destruction and osteophyte formation during osteoarthritis progression. Since the mechanism underlying these coordinated sequential steps remains an enigma, this study sought to identify the transcription factor and the related signals that control the stage.
Methods: A screen of transcription factors was performed using mouse chondrogenic ATDC5 cells and HeLa cells transfected with a luciferase-reporter construct containing a promoter of type X collagen (COL10), the marker for chondrocyte hypertrophy. The expression patterns were examined by immunohistochemistry of mouse growth plates and by real-time RT-PCR during insulin-induced differentiation of ATDC5 cells. Subcellular localization was examined by fluorescence microscope. Functional studies were performed using stable lines of ATDC5 cells with retroviral overexpression of HIF2A, and those with the dominant negative (DN) mutant or the small interfering RNA (siRNA). Transcriptional activity was determined by luciferase assay, and the specific binding between HIF2A protein and the identified region was verified by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay. Physiological function of HIF2A was assessed by histological comparison of the skeletal phenotypes of heterozygous HIF2A-deficient (HIF2A+/−) mice with those of the wild-type littemates (E16.5), since HIF2A−/− mice died in the early embryonic stage.
Results: Among over 100 transcription factors expressed in chondrocytes, hypoxia-inducible factor 2α (HIF2A), an α-unit member of the HIF family, most strongly stimulated the COL10 promoter activity. HIF2A was localized in the pre-hypertrophic and hypertrophic zones of the growth plate; and the expression increased during differentiation of ATDC5 cells in association not only with COL10 expression, but also with expressions of matrix metalloproteinase 13 (MMP13) and vascular endothelial growth factor (VEGF), crucial factors for matrix degradation and vascular invasion, respectively. Expressions of COL10, MMP13 and VEGF, as well as ALP and Alizarin red stainings, were enhanced by the HIF2A overexpression in ATDC5 cells, but were suppressed by the DN mutant or the gene silencing through siRNA. The promoter activities of the three genes were enhanced by the HIF2A overexpression. Deletion and mutation analyses identified the respective responsive elements, which were confirmed to show specific binding with HIF2A by EMSA and ChIP assay. The HIF family is known to work as a heterodimer of α-unit and β-unit members. Our search for the β-unit partner found that aryl hydrocarbon receptor nuclear translocator-like (ARNTL, also known as BMAL1), which was co-localized with HIF2A in the nucleus of differentiated chondrocytes, most strongly enhanced the transactivation of the three promoters by HIF2A, and was necessary for the specific bindings with the responsive elements and HIF2A. Finally, the HIF2A+/− mice were confirmed to exhibit dwarfism with impairment of the late stage of endochondral ossification in the growth plate cartilage.
Conclusions: HIF2A is the crucial transcription factor that controls the late stage of endochondral ossification through direct transactivation of COL10, MMP13 and VEGF. Elucidation of the signals related to HIF2A will lead to further understanding of the molecular background of osteoarthritis.

A5 AGE-RELATED CHANGES IN CHONDROCYTE DIFFERENTIATION MAKES CARTILAGE PRONE TO OA DEVELOPMENT

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Purpose: Chondrocytes in articular cartilage developing OA show deviant behavior. They seem to display a recapitulation of chondrocyte differentiation similar to chondrocyte hypertrophy in the growth plate. In the growth plate, Indian Hedgehog (Ihh) blocks terminal differentiation by keeping the chondrocytes in a pre-hypertrophic state. Loss of Ihh expression releases this block and allows the chondrocytes to undergo further differentiation. This led to the hypothesis that with age, which is the primary risk factor for OA, the expression of Ihh might be distorted in articular cartilage.
Methods: C57Bl/6 mice were sacrificed at 3, 6, 8, 10, 12, 14, 18, and 20 months of age. Knee joints were isolated for histology. Immunohistochemistry was performed staining the sections for Ihh. Tibial cartilage was scored for immunopositive cells using a computerized imaging system. We additionally studied Ihh expression by immunohistochemistry in cartilage during experimental OA induced in C57Bl/6 mice by either injection of collagenase (14, 21 and 42 days) or by destabilization of the medial meniscus (DMM model) (8 weeks). Ihh expression in spontaneous OA was analyzed in STR/ort mice aged 8 weeks and 3, 6, 9 and 12 months.
Results: Young mice expressed abundant Ihh in chondrocytes of the articular cartilage. With age the number of Ihh immunopositive cells rapidly declined. The number of Ihh positive cells declined 72% by 6 months of age compared to 3-month-old mice and declined even further after that. In lateral tibial cartilage the response was similar, but with a

lesser magnitude: a decline of 51% in 6-month-old compared to 3-month-old mice.
In the DMM model OA developed on the medial tibia where the number of Ihh expressing cells decreased 68% compared to sham operated individuals. The lateral tibial cartilage did not display a significant change in Ihh expression. In collagenase induced OA, Ihh expression decreased 18% on day 14, 42% on day 21 and 72% on day 42 in medial tibial cartilage compared to controls. On the lateral side this was 37%, 51% and 55% respectively.
In spontaneous OA (STR/ort), demonstrating mainly OA development at the medial tibia, a drastic change in Ihh expression was found with age and accordingly with OA in this age-related OA model. Whereas there is still normal Ihh expression at 8 weeks of age, it was almost absent by the age of 6 months in medial tibial cartilage and was reduced 54% in lateral tibial cartilage.
Conclusions: In the growth plate Ihh is known to keep chondrocytes in a pre-hypertrophic state by blocking further (terminal) differentiation. We found that with age there is a decrease in Ihh expression which is already found at an age of 6 months in C57Bl/6 mice. In earlier studies we showed that in normal articular chondrocytes there is high expression of TGF-beta signaling via the ALK5 receptor, leading to Smad2 phosphorylation. This pathway is known to block terminal differentiation in chondrocytes. During ageing and OA, this suppressive pathway of terminal differentiation is reduced.
In addition, we previously found that the balance between the ALK5 pathway and the alternative TGF-beta pathway via ALK1 leading to Smad1/5/8 phosphorylation is tilted during OA favoring the ALK1-side. This Smad1/5/8 pathway is a known stimulator of chondrocyte hypertrophy. Thus with age and OA we found a reduction in the block on terminal differentiation and even a shift towards stimulation of terminal differentiation. The results of this study, loss of Ihh expression during ageing and OA, appear to conform the hypothesis that chondrocytes undergo a shift in phenotype during ageing and OA that make these cells prone to terminal differentiation-like alterations and ageing cartilage to OA development.

A6 FUNCTIONAL CHARACTERIZATION OF TRPV4 AS AN OSMOTICALLY SENSITIVE ION CHANNEL IN ARTICULAR CHONDROCYTES

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Purpose: Transient receptor potential channel vanilloid 4 (TRPV4) is a calcium permeable cation channel that is gated by a number of factors, including osmolarity and temperature. Chondrocytes, the cells in articular cartilage, have been shown to perceive and respond to their osmotic and mechanical environments; however, the molecular basis of this signal transduction is not fully understood. The purpose of this study was to demonstrate the presence and functionality of TRPV4 in porcine articular cartilage.
Methods: TRPV4 protein expression was examined in porcine chondrocytes via Western blotting and immunostaining. TRPV4 mRNA was measured using RT-PCR. Calcium signaling in response to osmotic stress, TRPV4 agonist/antagonist, and various calcium signaling modifying agents was measured via fluorescent imaging of calcium-sensitive dyes. Cell volume change in response to osmotic stress, TRPV4 agonist/antangonist, and the inflammatory cytokine, Il-1, were measured from light microscopy images. Changes in prostaglandin E2 (PGE2), which is a downstream effect of increased internal calcium and Il-1, levels in response to osmotic stress and TRPV4 agonist/antagonist were measured with an immunoassay.
Results: TRPV4 was detected in porcine chondrocytes at the protein level and mRNA level. Addition of 4α-PDD, a TRPV4 agonist, caused calcium signaling, which was significantly blocked by the addition of ruthenium red (RR), a TRPV4 antagonist (Fig. 1). Calcium signaling was also inhibited by removal of extracellular calcium or blocking release from intracellular stores. Blocking TRPV4 with RR significantly modified the porcine cellular response to hypo-osmotic stress by decreasing the percentage of cells responding with a calcium signal. Addition of RR also significantly inhibited the volume recovery in response to hypo-osmotic stress, and 4α-PDD alone caused a slight volume increase (Fig. 2). TRPV4 activation was able to prevent the inflammatory cytokine Il-1 from inhibiting this volume regulation after exposure to hypo-osmotic medium (Fig. 2). Hypo-osmotic conditions caused PGE2 release which was blocked by the addition of RR (Fig. 3).

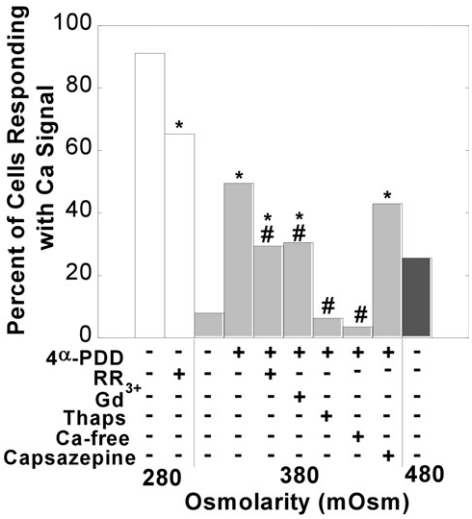

Fig. 1: Percentage of cells responding to stimuli with increases in intracellular calcium. Asterisks denote experimental groups that are significantly different from control within a given osmolarity (chi-squared test, $p < 0.05$); number signs specify bars within the 380 mOsm group that are significantly different from 380+4α-PDDm (chi-squared test, $p < 0.05$). Both the 280 and 480 mOsm controls are significantly different from the 380 mOsm control (chi-squared, $p < 0.05$).

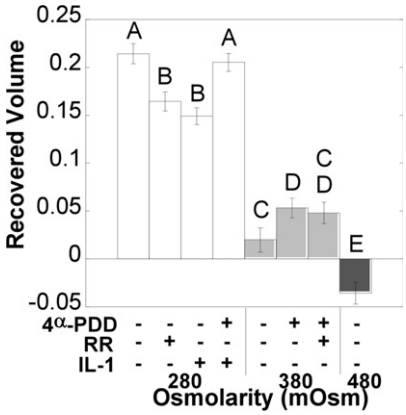

Fig. 2: Volume recovery (maximum-final) after stimulation with different osmotic and chemical stimuli. Bars are mean (±sem). Bars with different letters are significantly different from one another (ANOVA, $p < 0.05$).

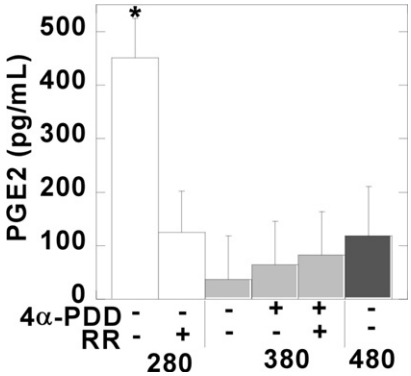

Fig. 3: Mean (+sem) levels of PGE2 in culture medium after treatment with different osmolarities and TRPV4 agonists/antagonists. Asterisk indicates bar is significantly different from all others (ANOVA, $p < 0.05$).

Conclusions: We have shown that TRPV4 is present in articular chondrocytes and that chondrocyte response to hypo-osmotic stress is me-